Development of the central nervous system in the larvacean *Oikopleura dioica* and the evolution of the chordate brain

Cristian Cañestro, Susan Bassham, John Postlethwait*

Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA

Received for publication 18 March 2005, revised 11 June 2005, accepted 17 June 2005

Available online 18 August 2005

Abstract

In non-vertebrate chordates, central nervous system (CNS) development has been studied in only two taxa, the Cephalochordata and a single Class (Ascidacea) of the morphologically diverse Urochordata. To understand development and molecular regionalization of the brain in a different deeply diverging chordate clade, we isolated and determined the expression patterns of orthologs of vertebrate CNS markers (*otxa*, *otxb*, *otxc*, *pax6*, *pax2/5/8a*, *pax2/5/8b*, *engrailed*, and *hox1*) in *Oikopleura dioica* (Subphylum Urochordata, Class Larvacea). The three *Oikopleura otx* genes are expressed similarly to vertebrate *Otx* paralogs, demonstrating that trans-homologs converged on similar evolutionary outcomes by independent neo- or subfunctionalization processes during the evolution of the two taxa. This work revealed that the *Oikopleura* CNS possesses homologs of the vertebrate forebrain, hindbrain, and spinal cord, but not the midbrain. Comparing larvacean gene expression patterns to published results in ascidians disclosed important developmental differences and similarities that suggest mechanisms of development likely present in their last common ancestor. In contrast to ascidians, the lack of a radical reorganization of the CNS as larvaceans become adults allows us to relate embryonic gene expression patterns to three subdivisions of the adult anterior brain. Our study of the *Oikopleura* brain provides new insights into chordate CNS evolution: first, the absence of midbrain is a urochordate synapomorphy and not a peculiarity of ascidians, perhaps resulting from their drastic CNS metamorphosis; second, there is no convincing evidence for a homolog of a midbrain–hindbrain boundary (MHB) organizer in urochordates; and third, the expression pattern of “MHB-genes” in the urochordate hindbrain suggests that they function in the development of specific neurons rather than in an MHB organizer.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Appendicularia; Forebrain; Midbrain; Hindbrain; Larvacea; Organizer; Chordate evolution of development; Tunicate; Subfunctionalization; Vertebrate; Urochordate; Rhombomere 4

Introduction

In vertebrate embryos, the expression of *Otx2* and *Hoxb1* in the dorsal epiblast reveals the nascent forebrain + midbrain and hindbrain before these regions become morphologically distinct (Lumsden and Krumlauf, 1996). Organizing centers then emerge along the anterior–posterior (AP) axis and play a crucial role in patterning the central nervous system (CNS). The isthmic organizer patterns the midbrain and hindbrain primordia (reviewed by Raible and Brand, 2004; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001), and the rhombomere-4 (r4) organizer patterns the surrounding hindbrain, at least in zebrafish (Maves et al., 2002; Walshe et al., 2002).

The isthmic (or midbrain–hindbrain boundary) organizer (MHB) develops in three phases: positioning, establishment, and maintenance (Rhinn and Brand, 2001). In the positioning phase, the MHB arises during gastrulation just anterior to the tip of the notochord between the posterior limit of the *Otx2* expression domain and the anterior limit of the *Gbx2* expression domain (Rubenstein et al., 1998). In the establishment phase, *Pax2*, *Fgf8*, and *Wnt1* expression initiates at the *Otx2–Gbx2* interface. During the maintenance phase, genes already used in the establishment phase and their downstream targets, including *En1*, *En2*, *Pax5*, and *Pax8*, become mutually dependent for their continued expression and maintain the boundary (Matsunaga et al., 2003).
amphioxus lacks an MHB (Holland et al., 1997; Kozmik et al., 1999). Caudal to the MHB, the presence of a second organizing activity in zebrafish (Maves et al., 2002), and possibly in other vertebrates (Graham et al., 1993, Graham and Lumsden, 1996, Marin and Charnay, 2000a,b) has been suggested by transplantation and ectopic expression studies; this activity is located in rhombomere 4 (r4) of the hindbrain, and patterns the surrounding rhombomeres by FGF signaling in the context of the Hox code.

Many genes with roles in the MHB subsequently play new roles in the progressive refinement of AP subdivisions and in the differentiation of specific cell populations in the CNS (Lumsden and Krumlauf, 1996). Most genes involved in CNS patterning belong to multigene families, many of which arose during large-scale gene duplication events that accompanied, and likely facilitated, early vertebrate evolution (Garcia-Fernández and Holland, 1994; Holland et al., 1994; Ohno, 1970). Understanding vertebrate CNS development is complicated because duplicated genes generally retain some redundancy but also acquire spatial and temporal diversification of expression patterns (Force et al., 1999; McClintock et al., 2002; Postlethwait et al., 2004).

Nervous systems of the non-vertebrate chordates—cephalochordates and urochordates—though simpler than the vertebrate CNS, share with vertebrates basic developmental genetic mechanisms (reviewed in Holland and Chen, 2001; Shimeld and Holland, 2000; Wada and Satoh, 2001). Understanding developmental mechanisms in non-vertebrate chordates, which possess low gene redundancy due to their divergence before the large-scale duplication events, can facilitate inferences for the roles of the founding members of CNS gene families in the last common ancestor of chordates. The Subphylum Urochordata, or Tunicata, is the sister taxon of the Cephalochordata + Vertebrata clade (but see Graham, 2004), and includes three Classes: ascidians, thaliaceans, and larvaceans. Among these, only ascidians have been intensively studied at the embryological and molecular level. The application of molecular and genomic tools has led to impressive progress in understanding ascidian development (reviewed in Cañestro et al., 2003; Corbo et al., 2001; Holland and Gibson-Brown, 2003; Jeffery, 2002; Lemaire et al., 2002; Meierntzhagen et al., 2004; Satoh, 2003; Sordino et al., 2001). Ascidians and amphioxus share with vertebrates the early expression of otx anteriorly and hox1 posteriorly, with an intervening gap. In vertebrates, the MHB forms in this gap. The role and fate of this gap in non-vertebrate chordates, however, are problematic (reviewed in Holland and Holland, 1999; Wada and Satoh, 2001). The discovery that pax2/5/8a is expressed in the otx–hox1 gap in the ascidian Halocynthia roretzi led to the proposal that the “neck” of the ascidian CNS is homologous to the MHB (Wada et al., 1998). In amphioxus, however, homologs of vertebrate MHB markers Pax2/5/8, Engrailed, and Wnt1 are not expressed in the otx–hox1 gap suggesting that amphioxus lacks an MHB (Holland et al., 1997; Kozmik et al., 1999). This apparent conflict was not resolved by recent studies of additional markers in ascidians, which revealed substantial variability in gene expression patterns among different ascidian species (Imai et al., 2002; Jiang and Smith, 2002; Mazet et al., 2003; Wada et al., 1998). The absence of functional data leaves unclear the roles of these genes during ascidian and cephalochordate CNS development, or whether any portion of the ascidian CNS possesses organizing activity.

Urochordate larvae possess the defining chordate features of notochord, dorsal hollow nerve cord, gill slits, and a muscular, post-anal tail. In ascidians, an elaborate metamorphosis erases most of these chordate features as the notochord is reabsorbed and the CNS is dramatically restructured. It is possible that selective pressures leading to rapid metamorphosis in ascidians have obscured or compromised some developmental mechanisms present in stem chordates. In contrast, larvacean urochordates (Class Appendicularia) retain a chordate body plan as adults. Phylogenetic relationships of urochordate classes are not yet known for certain, but the most accepted phylogeny places larvaceans as the basal sister group of ascidians + thaliaceans (Christen and Bracconnot, 1998; Holland et al., 1988; Swalla et al., 2000; Wada, 1998; Wada and Satoh, 1994; but see Stach and Turbeville, 2002). Genomic analysis has uncovered substantial differences in genome size and syntenic relationships between Oikopleura and ascidians (Edvardsen et al., 2005; Seo et al., 2001; Seo et al., 2004). For these reasons, the inclusion of larvaceans in developmental genetic investigations is essential for obtaining a balanced view of development in the urochordate Subphylum.

In the work reported here, we present the first molecular genetic analysis of larvacean brain regionalization. Significant homologies and differences between larvacean and ascidian CNS developmental mechanisms can be inferred from the comparison of gene expression patterns. Knowledge of Oikopleura CNS molecular regionalization provides new insights into chordate CNS evolution, helps illuminate previous conflicting interpretations of ascidian CNS expression data, extends within urochordates prior suggestions about CNS organization that previously applied only to ascidians, and provides a basis for functional genetic analysis.

Materials and methods

Animal culture, embryo staging, and imaging

Oikopleura dioica individuals were collected with plankton nets in the Pacific Ocean off Charleston, Oregon (Oregon Institute of Marine Biology) and Vancouver Island, B.C. (Bamfield Marine Sciences Centre). Animals were cultured for several generations in 10-μm filtered seawater in 4-l plastic jars and fed with algal concentrates (“Coral...
and Clam diet”, Reed Mariculture). Embryos were grown at 13 ± 3°C as described (Bassham and Postlethwait, 2000). Because developmental rate varies strongly with temperature [e.g., fertilization to hatching requires less than 3 h at 22°C, but about 12 h at 7°C (Fenaux, 1998)], we stage *Oikopleura* according to morphological landmarks. The
lack of a detailed blastomere fate map precludes an exact description of expression patterns at cleavage stages (Nishino and Satoh, 2001). Stages prior to hatching include: (i) incipient-tailbud stage (beginning about 3.5 h post-fertilization (pf) at 13°C), characterized by an indentation at the initial demarcation of trunk and tail (Fig. 1A) and by an irregular, rod-shaped notochord. (ii) Mid-tailbud stage (beginning about 4 h pf at 13°C), in which a deep indentation divides trunk and tail (Figs. 1B,C), and a rod-shaped row of 20 notochord cells is easily distinguishable by DIC microscopy (Fig. 1C). A twisting of the tail with respect to the trunk (Delsman, 1910) is already pronounced at this stage. This flexure makes it impossible to position embryos for a true orthogonal image capture, but the notochord and large muscle cells provide helpful landmarks to orient samples. Nuclear staining (i.e., DAPI, Hoechst) was routinely included in expression analysis at tailbud stages to confirm notochord cell positions.

Stages after hatching conform to Fenaux’s (1976), including: (i) early hatching (Fig. 1D) (Fenaux stage I), in which the animals are cylindrical, without substantial demarcation between trunk and tail, and, although the trunk still lacks obvious organs, the tail begins short bursts of movement; (ii) mid-hatching (Fenaux II) (Fig. 1E), in which the beginning of organogenesis is obvious, and animals can swim; (iii) late hatching (Fenaux IV) (Fig. 1F), in which movements of the heart and the cilia of the digestive system and spiracles become visible, the trunk-tail boundary is well defined, and coordinated swimming improves; (iv) tailshift, characterized by the shift of the tail to an acute angle relative to the trunk, signaling the end of embryonic development, and competence to secrete and inflate a filter-feeding house (Figs. 1G–J).

The transparency of Oikopleura embryos and adults allows non-invasive study of internal morphology at the level of individual cells. For some images, we merged DIC optical sections using Adobe-Photoshop software to integrate images of structures that spanned focal planes (Figs. 1D–J).

**Cloning and whole-mount in situ hybridization**

Genomic DNA from about 50 Oikopleura individuals from Bamfield Marine Station was used to construct an arrayed fosmid library (Epicentre, CCFOS110). Complete sequencing of targeted fosmid clones was performed by the DOE Joint Genome Institute (Walnut Creek, CA). mRNA from about 1500 embryos was used to synthesize cDNA as described (Bassham and Postlethwait, 2000). Genes of interest were amplified by PCR with degenerate primers (see Table S1 in supplementary materials) using cDNA or genomic DNA as template. Gene-specific primers were designed for RACE PCR, and to screen the genomic library using a pooling strategy. Coding sequence and gene structures were inferred by sequence comparisons between RACE products and fosmid genomic clones. We designate O. dioica genes by a name (usually based on its human ortholog) in italics and small-case letters (i.e., pax6), and proteins by a name with the first letter in upper-case (i.e., Pax6). When multiple paralogs are found, we add Latin letters in alphabetical order (i.e., otxa, otxb, otxc) with no additional punctuation. Whole-mount in situ hybridization was performed as described (Bassham and Postlethwait, 2000). Riboprobe information is provided in Table S2.

**Results**

**Isolation of Oikopleura CNS markers**

To study the development and regionalization of the larvacean CNS, we characterized CNS markers that are highly conserved across bilaterians and play a central role in AP organization of the tripartite vertebrate brain (Hirth et al., 2003; Reichert and Simeone, 2001). We isolated eight O. dioica genes homologous to the Otx, Pax6, Pax2/5/8, Engrailed, and Hox1 vertebrate gene families. Sequence comparisons and phylogenetic analyses unequivocally assigned each isolated Oikopleura gene to its gene family. We isolated single copies for Oikopleura pax6 (AY870650), engrailed (AY870647), and hox1 (AY871214) genes, three duplicated copies of otx (AY886542, AY897556, AY897557) and two copies of pax2/5/8 (DQ020279, AY870648, AY870649).

During the preparation of this manuscript, a list of O. dioica homeobox genes inferred from genome sequences was reported (Edwardsen et al., 2005), and in the present work, we have adopted the same names for the three Oikopleura otx duplicate genes we independently isolated: otxa, otxb, and otxc. Analysis of the predicted proteins for the three Oikopleura genes revealed the presence of a homeodomain, a single C-terminal hexapeptide motif (in contrast with two hexapeptide motifs in vertebrate OTX proteins), and a moderately conserved WSP motif (Fig. S1A). The small amount of evolutionary information provided by the conserved domains and variation in the rest of the molecule hampered the construction of confident protein alignments and phylogenetic inferences. Among the three Oikopleura Otx duplicates, Otxa showed the highest overall similarity to ascidian Otx. Analysis of exon–intron organization of Oikopleura otx genes revealed multiple introns in addition to the two introns conserved in all known otx genes (Fig. S1A). Remarkably, all ascidian and Oikopleura otx genes are characterized by the presence of additional exons (yellow in Fig. S1A) between the conserved exon containing the transcription origin and the exon containing the N-terminal part of the homeobox. The still detectable sequence similarities among these additional exons in urochordates suggest that these exons were originally gained before the separation of the ascidian and larvacean lineages; thus, they are likely a synapomorphy of urochordate otx genes. Analysis of fosmid sequences from
our *Oikopleura* genomic library (AY873983–AY873986) shows that *Oikopleura otxb* and *otxc* are adjacent, transcribed in opposite directions, and separated by a 17 kb stretch that contains no intervening putative genes. This result suggests that *Oikopleura otxb* and *otxc* probably arose by tandem gene duplication during the evolution of larvaceans after they separated from the ascidian lineage. Evidence from protein sequence similarities, gene structures, and phylogenetic analysis (data not shown and Edvardsen et al., 2005), shows that the duplication event that produced the third *Oikopleura otx* gene also probably occurred in the larvacean lineage after it separated from the ascidian lineage, although an *otx* gene lost during the evolution of the ascidian lineage cannot be ruled out.

We isolated from *Oikopleura* two *pax2/5/8* genes, only one of which (*pax2/5/8b*) was reported by Edvardsen et al. (2005). Sequence similarities and phylogenetic analysis showed that one *Oikopleura pax2/5/8* gene (AY870648, DQ020279) is orthologous to ascidian *pax2/5/8a* and the other (AY870649) is orthologous to ascidian *pax2/5/8b* (Fig. S1B). These results show that the duplication event that led to these two urochordate *pax2/5/8* clades occurred in the urochordate lineage after it diverged from the cephalochordate + vertebrate lineage, but before the split of the ascidian and larvacean lineages; therefore, the *pax2/5/8a+pax2/5/8b* duplicates appear to be a urochordate synapomorphy.

**Development and AP regionalization of the Oikopleura CNS**

Whole-mount in situ hybridization experiments revealed that the eight *Oikopleura* genes we isolated are transcribed in the embryonic CNS, endoderm, and epidermis. Here, we focus on expression patterns necessary to understand the mechanisms of CNS development (Figs. 2–4). The complex patterns of other expression domains will be described in detail elsewhere.

**Incipient-tailbud stage**

Analysis of the expression of *Oikopleura* CNS markers revealed that AP regionalization of the CNS begins early, probably during gastrulation. Expression of *otxb* and *hox1* is already detectable during cleavage stages (data not shown), and probably provides the initial AP information upon which subsequent AP regionalization is based.

At the incipient-tailbud stage, an anterior domain expresses *otxb* and a posterior domain in the presumptive CNS expresses *hox1* (Figs. 2A,G,P). In addition to epidermal expression, a broad *otxb* expression domain may span the entire presumptive anterior brain (Fig. 2A). At this stage, we did not detect expression of *otxa* or *otxc*.

At the incipient-tailbud stage, the presumptive anterior brain marked by *otxb* is subdivided by *pax6* expression, which appeared in two separate domains (Figs. 2D,F,P). At least two bilateral pairs of cells located at the midline constitute the most rostral *pax6* domain (Fig. 2D,d1). The caudal *pax6* domain, anterior to the tip of the notochord, also consists of at least two bilateral pairs of cells (Fig. 2D,d1,d2).

While *otxb* and *pax6* mark the anterior CNS, bilateral rows of cells express *hox1* dorsolaterally and posteriorly to the anterior tip of the notochord, at the level of the prospective caudal ganglion and anterior spinal cord (Figs. 2G,P).

Because cells expressing *otxb*, *pax6*, and *hox1* in the presumptive CNS were already internal and close to the midline at incipient-tailbud stage, we conclude that by this stage, neurulation is at or nearing completion.

Contrary to expectations, neither of the two *Oikopleura pax2/5/8* genes is specifically expressed in the CNS. *Pax2/5/8a* is expressed mainly in the trunk ectoderm (Fig. 2I). Complementary to *pax2/5/8a* expression, *pax2/5/8b* was detected in most of the internal portion of the trunk, probably including some presumptive CNS cells (Fig. 2M).

These diffuse *pax2/5/8* expression patterns are likely not artifactual since different non-overlapping probes render identical patterns, and the same probes reveal distinctive tissue-specificity at later stages (Fig. 2K and data not shown).

**Mid-tailbud stage**

At mid-tailbud stage, early broad gene expression domains become refined and the expression of new CNS
markers appears. At this time, while \textit{otxb} continues to label a row of cells in the midline of the presumptive anterior brain, \textit{pax6} expression is reduced to a single domain (Figs. 2B,E,Q), apparently by down-regulation of the more posterior of the two domains seen in incipient-tailbud embryos. At mid-tailbud stage, \textit{otxa} expression was
detected for the first time in the CNS, labeling asymmetrically one cell in the right side and at least four cells in the left side of the presumptive anterior brain (Fig. 2C). At this stage, otxc expression was not yet detected within the CNS.

The posterior CNS becomes subdivided in mid-tailbud embryos, with the appearance of a hox1–engrailed–hox1 nested pattern. Analysis of this nested pattern by single and double in situ hybridization experiments, together with notochord cell positioning by detection of brachyury expression (Bassham and Postlethwait, 2000) and nuclear staining (Figs. 2H,I,L,O,Q,R), revealed an anterior hoxl domain spanning approximately from the AP level of the staining (Figs. 2H,I,L,O,Q,R), revealed an anterior hoxl expression (Bassham and Postlethwait, 2000) and nuclear

brachyury notochord cell positioning by detection of double in situ hybridization experiments, together with nested pattern. Analysis of this nested pattern by single and

hox1 – otx4 expression fills the entire presumptive anterior brain (Fig. 2A) to span the entire presumptive anterior brain (Fig. 2A) to

prominent expression of otxc in the anterior brain, outside the future sensory vesicle (Figs. 3A,B). A thin extension of expression reaches forward from pax6-positive cells (rostral arrowhead in Fig. 3A), suggesting the formation of neuronal processes. The internal pax2/5/8a expression domain observed anterior to pax6 expression at mid-tailbud stage disappears by mid-hatchling stages (data not shown).

Otcb expression narrows in hatchlings, from initially spanning the entire presumptive anterior brain (Fig. 2A) to comprising only a few cells (Fig. 3D). Interestingly, as otcb expression declines in early hatchlings (Fig. 3D), the otcb expression signal becomes intense (Fig. 3G), and for the first time, otxc transcripts begin to accumulate in CNS cells (Fig. 3J). This process, in which otcb + otxc expression seems to replace otcb expression over time, is finished by late hatchling stages when otcb transcription is almost undetectable in the anterior brain (Fig. 3F).

During hatchling stages, the expression of the three otv duplicates is restricted to the central portion of the anterior brain, although there are differences in their expression patterns; while some cells apparently co-express multiple otv duplicates, other cells express only one, and in some cases the expression patterns are asymmetric (Figs. 3D,E,G,H,J,K).

In early hatchling stages, in addition to strong epidermal hoxl expression at the trunk-tail transition (Fig. 4A, black arrowheads), a row of hoxl-expressing cells extends left of the midline from anterior positions near the notochord tip (within the trunk) caudally to at least the level of the fourth notochord cell (Figs. 4A,B), probably spanning the prospective posterior TNC and anterior caudal ganglion (Fig. 1D). Additional hoxl signal with irregular shape and thin diameter appears at the level of the fifth and sixth notochord cells (Figs. 4A,B), and could mark caudal cellular extensions from anterior hoxl-expressing cells. These observations broadly confirm and extend those for hoxl in Seo et al. (2004). In early hatchlings, in addition to the expression of hoxl in the anterior segment of the caudal ganglion, we observed expression of engrailed (Fig. 3)}
4D), and, transiently, pax6 (Fig. 3A) posterior to the notochord tip.

In mid-hatchling stages, while precursor cells of the posterior TNC continue to express hox1, the prospective caudal ganglion down-regulates hox1 expression (Fig. 4B). We found no expression of hox1 in the spinal cord, although punctate, non-epidermal hox1 and engrailed signal appears broadly distributed along mid- and late-hatchling tails (Figs.
4B,E,F, white arrowheads). Although this punctate pattern is specific for these two genes, the pattern varies in different animals.

Late-hatchling stage

In late hatchlings, the expression pattern of CNS markers mapped to the four morphologically distinct regions of the CNS, the anterior brain (AB), trunk nerve cord (TNC), caudal ganglion, and spinal cord (Figs. 3–5), and they divide the anterior brain into three subdivisions (AB₁–₃) (Figs. 5A,B). AB₁, the anterior subdivision, is labeled by \( \text{pax6} \) (Fig. 3C). The ciliary funnel and the anterior extensions from AB₁ that form the rostral paired nerve n₁ appear to be free of \( \text{pax6} \) expression. AB₂, the central subdivision, is broadly marked by the expression of \( \text{pax6}, \text{otxa}, \) and \( \text{otxc} \) (Figs. 3C, I, L). While some cells appear to co-express \( \text{pax6}, \text{otxa}, \) and \( \text{otxc} \) genes, the most dorsal AB₂ cells appear not to express \( \text{otx} \), and the most ventral cells appear not to express \( \text{pax6} \). In addition, the statocyte expresses \( \text{otxa} \), but not \( \text{pax6} \) or \( \text{otxc} \) (Figs. 3C, I, L). AB₃, the posterior subdivision, was free of any \( \text{pax6}, \text{otx}, \text{engrailed}, \text{pax2/5/8}, \) and \( \text{hox1} \) signal (Figs. 3C, FI, L, 4C, F, and 5A). These three subdivisions of the anterior brain broadly coincide with regions of the \( \text{Oikopleura} \) brain previously called fore-, mid-, and hind-brain, based on fine structure and nerve positions (Olsson et al., 1990). Calling these regions AB₁₋₃ avoids potentially misleading homologies with the vertebrate brain (see Discussion).

In the posterior trunk of late hatchlings, the TNC bends 90° towards the caudal ganglion (Fig. 1F). Only the posterior half of the TNC is labeled by \( \text{hox1} \) expression (Figs. 4C and 5A). \( \text{Hox1} \) signal was also detected in the sensory field that includes the epidermal Langerhans mechanoreceptors (Fig. 4C), which are innervated by axons from the caudal ganglion (Bone and Mackie, 1975). At this late stage, the caudal ganglion itself does not express \( \text{hox1} \), and among the genes we studied, only \( \text{engrailed} \) expression appeared in the anterior part of the caudal ganglion (Figs. 4C, F).

Discussion

Homologies and differences between larvacean and ascidian CNS development

This study of gene expression in the developing \( \text{Oikopleura} \) CNS reveals the molecular genetic regionalization of the larvacean CNS, and allows comparison...
with ascidians. The ascidian CNS has been divided into four regions: the "brain" (which includes the sensory vesicle housing the statocyte and ocellus), neck, visceral ganglion, and spinal cord (Satoh, 2003). The following section highlights evidence that defines regional homologies in the CNS of two urochordate classes, and discusses the differences observed along the AP axis of the CNS, starting rostrally and moving caudally.

**The anterior CNS**

The Oikopleura anterior brain and ascidian "brain" are homologous. Expression of *Oikopleura otxb* and ascidian *otx* demarcates the anterior CNS in cleavage stages (Hinman and Degnan, 2000; Hudson and Lemaire, 2001; Wada et al., 1996; Wada et al., 2004; and this study). Both urochordate classes have two *pax6* expression domains, the posterior of which overlaps the expression of *otx* near the
Expression of *otx* and *pax6* reveals differences between the *Oikopleura* anterior brain and the ascidian “brain”. Although early expression of *otx* and *pax6* is similar in the two urochordate classes, at later stages, there are three major differences. First, *pax6*, which is typically expressed in photoreceptors (Callaerts et al., 1997), is expressed in the photosensitive ocellus in the ascidians (Glardon et al., 1997), but not in that of *Oikopleura*, which lacks pigmented photoreceptor cells. The second major difference is that while *pax6* is no longer expressed in the ascidian “brain” by late larval stages (Mazet et al., 2003), in *Oikopleura*, it continues to be expressed in the anterior brain until at least the late hatchling stage (Figs. 3C and 5A,C,e). This expression difference perhaps reflects the continuity of embryonic and adult *Oikopleura* CNS patterning (see discussion below), while the ascidian CNS is drastically restructured during metamorphosis.

The third major difference is that the three *Oikopleura* *otx* genes, in contrast to the single ascidian *otx* gene, show “phased” expression dynamics, differing in their temporal and spatial expression patterns during CNS development. These results suggest the hypothesis that the three *otx* genes in *Oikopleura* have different developmental functions. The broad and uniform expression of the single *otx* gene described in the ascidian brain (Wada et al., 1996) would mask potential multiple functions. Like *Oikopleura*, tetrapods have three *OTX* paralogs (*OTX1*, *OTX2*, and *OTX5/CRX*) due to independent duplications within the vertebrate lineage. The vertebrate paralogs, like their *Oikopleura* homologs, have also assumed separate early and late developmental roles. First, the earliest expression of *Oikopleura otxb* in the anterior neuroectoderm and endoderm is comparable to the expression of *OTX2* during vertebrate gastrulation (Reichert and Simeone, 2001). And second, the later appearance of *Oikopleura otxa* and *otxb* expression in a reduced number of cells in the anterior brain can be compared to the action of *OTX1* and *OTX2* in specifying identity and fate of specific cell populations in the vertebrate brain (Puelles et al., 2003; Puelles et al., 2004). This parallelism between vertebrates and *Oikopleura* provides an example of trans-homologs [genes duplicated independently in different lineages from a common ancestral pro-ortholog (Sharman, 1999)] converging on similar evolutionary outcomes. Functional analysis of *otx* regulation in *Oikopleura* duplicates will test whether this evolutionary convergence is due to parallel subfunctionalization events in conserved regulatory modules (Force et al., 1999) present in the last common ancestor of vertebrates and urochordates, or whether phased *otx* regulation has been gained independently in the two chordate lineages.

Pax2/5/8 genes reveal differences between *Oikopleura* and ascidian anterior CNS regions. In ascidians, the expression of *pax2/5/8a* in the gap between *otx* and *hox1* domains suggested homology with the vertebrate MHB (Wada et al., 1998). Despite the expression similarities between *Oikopleura* and ascidian *pax2/5/8a* and *pax2/5/8b* orthologs in several domains (Mazet et al., 2003; Wada et al., 1998), neither *Oikopleura pax2/5/8a* gene is expressed in the gap between *otx* and *hox1* domains. Therefore, *pax2/5/8* expression data argue against the presence of an MHB homolog in *Oikopleura*.

In mid-tailbud stages, ascidians lack *pax2/5/8a* expression similar to the *Oikopleura pax2/5/8a* internal domain at the brain–pharynx border anterior to the *pax6* domain (Mazet et al., 2003; Wada et al., 1998). In amphioxus, *pax2/5/8* expression in the anterior cerebral vesicle has been compared to vertebrate *Pax2* expression in development of the optic stalk and optic nerve (Fig. 5C.a) (Kozmik et al., 1999; Krauss et al., 1991; MacDonald et al., 1997). Additionally, it was proposed that *pax2.1*, in cooperation with Fgf signaling, influences axon guidance and early rostral midline development in the zebrafish forebrain (Shanmugalingam et al., 2000). Because AB1, the rostral subdivision of the anterior brain of *Oikopleura*, projects axons rostrally via the paired nerves n1 and n2 to the ventral organ and sensory receptors in the lips (Figs. 11 and 5B), *Oikopleura pax2/5/8a* expression is consistent with a role in axon guidance like its vertebrate homolog *Pax2*. Study of more markers and gain- and loss-of-function experiments are necessary to test these hypotheses for the role of the *Oikopleura pax2/5/8a*.

Links between developmental expression patterns and the adult larvacean brain. In contrast to ascidians, the continuous transition of the *Oikopleura* CNS from hatching to adult provides direct links between embryonic gene expression domains and the fine structure and function of the adult *Oikopleura* brain (Olsson et al., 1990). Figs. 5A,B show the major anatomical features of the anterior CNS in the juvenile and adult *Oikopleura*. The rostral subdivision of the anterior brain (AB1) labeled by *pax6* expression receives afferent pathways from anterior sensory cells, including the ventral organ (via n1), and ciliated receptor cells in the lips and pharynx (via n2) (Olsson et al., 1990; and Fig. 5B). The efferent pathways described by Olsson et al. (1990) may originate in the central and posterior anterior tip of the notochord (Figs. 2P and 5C.c.f) (Glardon et al., 1997; Mazet et al., 2003). By the mid-tailbud stage in *Oikopleura*, although the *otxb* posterior boundary does not change relative to the notochord, *pax6* is expressed only in the former anterior domain, suggesting down-regulation of *pax6* expression in the posterior domain (Figs. 2Q and 5C.d). Similarly, *pax6* expression is absent from the posterior part of the ascidian brain at mid-tailbud stage (Glardon et al., 1997; Mazet et al., 2003). This congruence of *otx* and *pax6* expression patterns in the early tailbud stages of *Oikopleura* and ascidians supports homology of the larvacean anterior brain and the ascidian “brain”.

The common ancestral pro-ortholog (Sharman, 1999) converging on similar evolutionary outcomes. Functional analysis of *otx* regulation in *Oikopleura* duplicates will test whether this evolutionary convergence is due to parallel subfunctionalization events in conserved regulatory modules (Force et al., 1999) present in the last common ancestor of vertebrates and urochordates, or whether phased *otx* regulation has been gained independently in the two chordate lineages.
subdivisions AB2 and AB3. AB2, which is labeled by pax6, otxα, and otxβ, probably includes three neurons that project axons via n3, a postulated motor nerve carrying fibers to the spiral's ciliary rings and to ventral epidermal cells that might have a role in house secretion (Fig. 5B). The posterior subdivision AB3, which does not express any of the genes in this study during late development, sends one process via the left n3 to the ciliary ring and another into the TNC, probably towards the caudal ganglion. Therefore, the AB1–3 subdivisions revealed by molecular markers during development seem to correspond to different functional areas of the brain in charge of integrating afferent and efferent pathways.

The posterior CNS

The posterior urochordate CNS features a prominent ganglion near the anterior tip of the notochord called the caudal ganglion in Oikopleura and the visceral ganglion in ascidians (Fig. 1) (Meinertzhagen et al., 2000). Variation in gene expression and morphology among ascidian species, however, hinders a straightforward assignment of homology between larvacean and ascidian posterior CNS regions.

Larvaceans and ascidians share a two-domain expression profile for hox1 (Fig. 5C,d,g): an anterior domain near the anterior tip of the notochord [compare dorsal views of Oikopleura in Fig. 4A to views of the ascidian in Fig. 5 of Katsuyama et al. (1995)] and a posterior, transient domain in the presumptive caudal/visceral ganglion and spinal cord (Figs. 2P–Q and 5A,C) (Ikuta et al., 2004; Katsuyama et al., 1995; Nagatomo and Fujiwara, 2003). In Oikopleura, we can trace the anterior hox1 domain throughout development to the posterior TNC. In ascidians, although the hox1 expression pattern is the same in different species, various authors have differed in their interpretation of the fate of the anterior domain, assigning it either to the neck or to the visceral ganglion (Ikuta et al., 2004; Katsuyama et al., 1995; Nagatomo and Fujiwara, 2003; Wada et al., 1998). This controversy is probably due to the lack of a morphologically distinguished neck in H. roretzi rather than a true fate difference.

These expression data also indicate shared morphogenetic behaviors of larvacean and ascidian axial tissues. The anterior hox1 expression domain shifts forward with respect to the notochord during Oikopleura development, as it does in ascidians (Katsuyama et al., 1995). This domain shift probably explains the variability of the cell positions of the anterior hox1 domain observed in Oikopleura mid-tailbud stages as different individuals are fixed at slightly different stages (Figs. 2H–I,R). In Ciona intestinalis, neck precursor cells apparently move from behind the tip of the notochord in tailbud embryos to an anterior location in hatchlings, suggesting that the neck is part of the visceral ganglion (Cole and Meinertzhagen, 2004; Meinertzhagen et al., 2000). This shift applies also to H. roretzi pax2/5/8a-positive cells (Wada et al., 1998). Therefore, despite the morphological differences between the CNS of the two urochordate classes, similar morphogenetic processes seem to be conserved underlying the development of the neural tube at the level of the anterior tip of the notochord.

Engrailed expression in the posterior CNS is also similar among urochordates. In Oikopleura, engrailed expression begins at mid-tailbud stage between the two initial hox1 domains creating a hox1−engrailed−hox1 nested pattern (Fig. 2R). Throughout development, engrailed expression continues posterior to the anterior notochord tip, labeling the anterior part of the caudal ganglion (Figs. 2L,O,R and 4D–F). Expression of engrailed differs between the two congeneric ascidian species in which it has been described. In Ciona savignyi, a single bilateral pair of cells co-expresses engrailed and pax2/5/8a (Jiang and Smith, 2002). In the congener C. intestinalis, pax2/5/8a is also expressed in a single pair of cells, but engrailed flanks the pax2/5/8 expression domain in an engrailed-pax2/5/8a-engrailed nested pattern (Fig. 5C.g), in which the posterior engrailed domain labels the anterior part of the visceral ganglion (Imai et al., 2002). Although no direct comparison of hox1 and engrailed expression has been published for any ascidian, comparing the positions of hox1 and engrailed expression domains with the notochord (Ikuta et al., 2004; Imai et al., 2002; Nagatomo and Fujiwara, 2003) makes it likely that the hox1−engrailed−hox1 nested pattern is shared by Oikopleura and at least some ascidians (Fig. 5C.g). The fact that pax6 is expressed transiently at early hatching stages in the presumptive caudal/visceral ganglion approximately at the same level as engrailed (Glardon et al., 1997; Mazet et al., 2003; and our Fig. 3A) bolsters the correspondence of larvacean and ascidian posterior engrailed domains.

The similar expression of engrailed, pax6, and hox1 during development of the caudal ganglion in Oikopleura and the visceral ganglion in ascidians suggests that these two structures are homologous. The present expression data are also consistent with the homology of the ascidian neck and the posterior Oikopleura TNC. In agreement with the interpretation that the ascidian neck is part of the visceral ganglion (Meinertzhagen et al., 2000), we conclude that the posterior TNC and the caudal ganglion derive from the same embryonic CNS region labeled by the single hox1 domain at incipient-tailbud stage (Fig. 2G). Because Oikopleura engrailed expression remains in a constant position posterior to the tip of the notochord, while the hox1 domain shifts anteriorly, there may also be elongation or migration of specific cells, or stationary cells turning on and off expression, rather than simply a general anterior shift of this entire part of the posterior CNS.

Homologies and differences between urochordate and vertebrate CNS development

Our comparative analysis of Oikopleura and ascidian urochordates has revealed homologies and differences in
developmental genetic pathways, which now can be compared to other chordate Subphyla.

Which part of the vertebrate CNS is homologous to the urochordate spinal cord?

In contrast to ascidians and vertebrates, _Oikopleura_ and cephalochordates (Glardon et al., 1998) lack _pax6_ expression along the length of their spinal cords. Because the posterior _Drosophila_ CNS expresses _ey_, which is the fly ortholog of _pax6_ (Quiring et al., 1994), the most parsimonious explanation for species-specific _pax6_ expression patterns is that the last common ancestor of extant chordates had _pax6_ expression in the spinal cord and it was independently lost in the _Oikopleura_ and cephalochordate lineages.

The similar expression pattern between ascidian _hox5_ (Gionti et al., 1998) and the only _Oikopleura_ central _Hox_ subclass gene (called _hox4_, although equally related to _hox4/5/6/7_) (Seo et al., 2004) suggests that these genes may function to define the anterior boundary of the urochordate spinal cord. Therefore, despite the variation in _pax6_ expression patterns among chordates, we conclude that the spinal cord of urochordates and vertebrates is homologous.

Which part of the vertebrate CNS is homologous to the urochordate TNC/neck and caudal ganglion/visceral ganglion?

Since the caudal ganglion of _Oikopleura_ and the ascidian visceral ganglion are probably homologous, as are the posterior TNC in _Oikopleura_ and the ascidian neck, and since these structures derive from a posterior CNS region expressing _hox1_, we conclude that these structures are homologous to at least part of the vertebrate hindbrain (Fig. 5C.b,c). The presence of motor function in the vertebrate hindbrain (Lumsden and Krumlauf, 1996), the existence of motor neurons in the caudal ganglion that coordinate muscular tail movements in larvaceans (Bone, 1998), and the presence of motor neurons in the neck and visceral ganglion of ascidians (Katsuyama et al., 2005; Meinertzhagen et al., 2000; Okada et al., 2002) are consistent with this proposed homology; we will therefore refer to the posterior-TNC/neck plus caudal/visceral ganglion as the "urochordate hindbrain".

In ascidians, _hox3_ expression suggests that the anterior limit of the visceral ganglion corresponds to the anterior limit of r4 of the vertebrate hindbrain (Locascio et al., 1999). Unexpectedly, despite other gene expression similarities between the larvacean caudal ganglion and the ascidian visceral ganglion (Fig. 5C.d,g), there does not appear to be a _hox3_ ortholog in the _Oikopleura_ genome (Seo et al., 2004). Analysis of additional hindbrain markers such as Kreisler and Krox20 could help us understand the consequences of the loss of _Oikopleura hox3_.

Which part of the vertebrate CNS is homologous to the urochordate "anterior brain"?

In addition to the "urochordate hindbrain", we designate the anterior part of the urochordate CNS as "anterior brain", rather than just "brain", because in vertebrates the term "brain" includes the forebrain, midbrain, and hindbrain.

Data from ascidians have led to conflicting interpretations concerning homologies between the ascidian "brain" and the vertebrate brain, sometimes because of differences in morphology and gene expression among ascidian species (Lemaire et al., 2002; Locascio et al., 1999; Meinertzhagen and Okamura, 2001; Meinertzhagen et al., 2004; Satoh, 2003; Takahashi and Holland, 2004; Wada and Satoh, 2001). For example, the posterior part of the ascidian "brain" has inconsistently been proposed to be homologous to (i) the vertebrate metencephalon [based on the co-expression of _Ci-otx_ and _Ci-engrailed_ (Meinertzhagen et al., 2004)]; (ii) the vertebrate midbrain [based on the co-expression of _Ci-otx_ and _Ci-engrailed_ (Imai et al., 2002)]; or (iii) the vertebrate forebrain [based on the presence of _Ci-otx_ expression (Hudson and Lemaire, 2001) and the absence of _Ci-dmbx_ expression (Takahashi and Holland, 2004)]. To address these conflicting conclusions, we first integrated larvacean and ascidian CNS gene expression patterns (see above), and now we compare that result to vertebrate expression patterns.

In vertebrates, forebrain and midbrain are labeled by _Otx_ expression (Fig. 5C.b). The expression of _Pax6_ in two domains, one in the posterior forebrain and one in the anterior hindbrain, has been used to define the midbrain, which develops in the intervening gap ("pax6-gap") and is regulated by _Pax2_ and _Engrailed_ expression (Fig. 5C.b) (Matsumaga et al., 2000; Scholpp et al., 2003; Schwarz et al., 1999).

As in vertebrates, the prospective anterior brain area of urochordates is labeled by _otx_ expression (Fig. 5C.e,f), suggesting at first glance that the urochordate anterior brain is homologous to the vertebrate forebrain + midbrain (Fig. 5C.b). In urochordates, however, the fact that the _otx_ domain is subdivided along the AP axis by two expression domains of _pax6_ at early tailbud stage (Fig. 5C.c,f), leads to an alternative interpretation. In this alternative, the anterior _pax6_ domain in urochordates labels the homolog of the vertebrate forebrain, while the posterior _pax6_ domain labels the homolog of the anterior hindbrain, and the _pax6-gap_ could be the urochordate homolog of the vertebrate midbrain. This interpretation, however, conflicts with two facts. First, the _pax6-gap_ of the urochordate anterior brain fails to express the vertebrate midbrain markers _pax2/5/8_ and _engrailed_ (Fig. 5C.b,c,f). And second, the posterior expression domain of _pax6_ in the urochordate anterior brain overlaps the _otx_ expression domain, while _Otx_ expression is excluded from the vertebrate hindbrain (Fig. 5C.b,c,f). Therefore, these data lead to the conclusion that the urochordate anterior brain is homologous to the vertebrate forebrain (Fig. 5C.b,c).
New perspectives on chordate CNS evolution from an Oikopleura point of view

GbX gene loss, an ancestral event in urochordate evolution

In vertebrates, the action of Gbx excludes the posterior boundary of Otx expression from the anterior hindbrain labeled by Pax6 (Fig. 5C.b) (reviewed in Rhinn and Brand, 2001). We know that the boundary between Otx and Gbx expression domains is crucial to define the limit between the midbrain and the hindbrain in vertebrates because vertebrate Gbx mutants show caudal expansion of Otx expression back to the level of rhombomere 4. The finding that Gbx also antagonizes Otx in Drosophila suggests that this gene interaction is ancient in Bilateria (Hirth et al., 2003; Reichert and Simeone, 2001). Interestingly, the apparent absence of any Gbx homolog from the genome projects of C. intestinalis (Wada et al., 2003) and C. savignyi (http://www.broad.mit.edu/annotation/ciona/index.html), and the failure to isolate a Gbx homolog from Oikopleura [despite our unpublished efforts and the genome sequencing traces (Edvardsen et al., 2005)], strongly suggest that the loss of Gbx is a urochordate synapomorphy. This gene loss might have affected the evolution and regionalization of the urochordate CNS, perhaps by permitting a caudal expansion of the otx domain as observed in vertebrate Gbx mutants. A caudal expansion of otx expression in an ancestral urochordate would be consistent with our comparative analysis of Oikopleura and ascidian data, which shows that the posterior boundary of otx expression overlaps with the posterior pax6 expression domain in the anterior brain (see discussion above and Fig. 5C.b,c,f). The study of additional urochordate orthologs of genes upstream and downstream of Gbx function in the vertebrate anterior hindbrain (e.g., Wnt1, Fgf8) will help assess the impact of the loss of gbx from the urochordate genome.

Do urochordates have a homolog of the vertebrate midbrain?

In the Oikopleura CNS, absence of engrailed and pax2/5/8 expression anterior to the hox1 expression domain that labels the hindbrain argues against the presence of midbrain in the larvalence lineage. Thus, we conclude that the absence of a midbrain in ascidians, recently proposed by T. Takahashi and P.W.H. Holland (2004) based on the absence of dmxb expression in the anterior brain, is not just a peculiarity of the ascidian Class, resulting perhaps from its drastic CNS metamorphosis, but is a synapomorphy of the urochordate Subphylum.

Absence of evidence supporting the existence of a midbrain in amphioxus (Holland et al., 1997) and ascidians (Takahashi and Holland, 2004) led to the most parsimonious conclusion that the absence of a distinct midbrain was the ancestral condition for chordates, and that the midbrain is a developmental innovation in the vertebrate lineage (Takahashi and Holland, 2004). Although this conclusion continues to be the most parsimonious explanation, our data on Oikopleura CNS are also consistent with a different hypothesis. The posterior overlap of otx and pax6 expression, the absence of gbx in urochordates, and the variability or absence of the expression of orthologs of vertebrate midbrain markers (such as engrailed and pax2/5/8) in the urochordate anterior brain, suggest that the midbrain was present in the last common ancestor of extant chordates, but was modified or lost independently in the urochordate and in the cephalochordate lineages. Study of the developmental mechanisms underlying the posterior subdivision of the anterior brain of Oikopleura (AB3) could provide new data to better understand the evolution of the urochordate brain, because AB3 is located just anterior to the urochordate hindbrain, and its homology is as yet uncertain due to the absence of expression of all CNS markers so far analyzed.

Do urochordates have a midbrain–hindbrain organizer (MHB)?

In vertebrates, the MHB forms at the border of Otx and Gbx expression domains, and requires expression of Pax2, Engrailed, Wnt11, and Fgf8 genes (reviewed in Rhinn and Brand, 2001). The absence of a Gbx ortholog in urochordates makes it difficult to recognize a putative MHB organizer in this Subphylum. In Oikopleura, the absence of engrailed and pax2/5/8 expression immediately posterior to the otx expression domain argues against the presence of an MHB organizer homolog in the larvalence CNS. In ascidians, it was argued that because pax2/5/8 expression in the neck coincides with the gap between otx and hox1 expression domains (Wada et al., 1998), and because engrailed and fgf8/17/18 expression appears in the anterior visceral ganglion (Imai et al., 2002), the neck region of the ascidian CNS is homologous to the vertebrate MHB (Imai et al., 2002; Jiang and Smith, 2002; Meinertzhagen et al., 2004; Satoh, 2003; Wada et al., 1998; Wada and Satoh, 2001). Close inspection of Ci-pax2/5/8, Ci-engrailed, and Ci-fgf8/17/18, however, reveals that they are not co-expressed in C. intestinalis (Imai et al., 2002). If these genes are not co-expressed, then they cannot have the same genetic interactions their orthologs have in the development of the vertebrate MHB. Even if Ci-fgf8/17/18 signaling might provide organizer function in the anterior part of the visceral ganglion in ascidians, that function might not necessarily be homologous to the MHB organizer specifically, because Fgf8 is also active in the r4 organizer in the hindbrain of the vertebrate Danio rerio (Maves et al., 2002; Walshe et al., 2002). The interpretation that Fgf expression in the ascidian CNS could be homologous to the r4 organizer is compatible with our definition of the “urochordate hindbrain”, and with the correspondence of the anterior part of the visceral ganglion in ascidians with rhombomere 4 in vertebrates based on expression of Ci-hox3 (Locascio et al., 1999).

Finally, the obvious differences in the expression patterns of engrailed and pax2/5/8a between Oikopleura and
ascidians, and indeed among different ascidian species [i.e., even between the congeners C. savignyi and C. intestinalis (Imai et al., 2002; Jiang and Smith, 2002)], are not expected if the presence of an MHB organizer is fundamental for the regionalization of at least the ascidian CNS. Therefore, in the light of our data from Oikopleura and published results in ascidians, and in the absence of any functional data about the roles of engrailed, pax2/5/8, and fgf8/17/18 in ascidians, we conclude that there is no convincing evidence of an MHB homolog in urochordates. Characterization and functional analysis of Fgf family members in Oikopleura and ascidians would help to test whether organizer activity exists in urochordates.

**Alternative hypothesis for the function of “MHB genes” in urochordates**

Urochordate tailbud stage embryos may correspond to much later developmental stages than the vertebrate gastrula and neurula stages in which the MHB forms. In vertebrates, many of the genes that are involved in the development of the MHB are also expressed later in the hindbrain, where they perform different functions than they do in the MHB (Lumsden and Krumlauf, 1996). In the vertebrate hindbrain, Pax2, Pax5, Pax8, En1, En2, Pax6, Fox1, Dmbx1, and Lim transcription factors, in conjunction with graded FGF signals, specify motor neurons and interneurons, and are important for axon guidance (Burriil et al., 1997; Dasen et al., 2003; Gavalas et al., 2003; Irving et al., 2002; Kawahara et al., 2002; Pfeiffer et al., 1998; Sapir et al., 2004; Segawa et al., 2001). In cephalochordates, the expression of pax2/5/8 and en that appears during late developmental stages in the amphioxus hindbrain, has been postulated to be related to functions in neuron specification (Holland and Holland, 1999; Holland et al., 1997; Kozmik et al., 1999). The late expression of “MHB genes” in the vertebrate and cephalochordate hindbrains raises the hypothesis that tailbud stage expression of the orthologs of these transcription factors in the “urochordate hindbrain” is related to specification of neuron fate and axon guidance rather than an organizer function.

This alternative hypothesis is consistent with several facts. First, shortly after hatching, Oikopleura and ascidians probably have functional motor neurons because they show coordinated tail movement. Second, motor neurons confined to the ascidian CNS neck region begin to be specified as early as late-gastrula stage (Katsuyama et al., 2005; Okada et al., 2002). And third, the differences in expression patterns of “MHB genes” in tailbud embryos between different ascidian species, and between Oikopleura and ascidians could merely reflect species-specific modifications of developmental time and embryonic position of specific neurons in the urochordate hindbrain rather than fundamental differences in CNS regionalization. Taken together, these considerations suggest that the expression of “MHB genes” described so far in Oikopleura and ascidians reflects development of specific neurons rather than action in an MHB organizer. Functional experiments will be necessary to disprove this conclusion.

**Acknowledgments**

We are grateful to Skipper B. Young of the “Charming Polly” for help in larvacean collecting. We thank F. Mazet for sharing with us her unpublished results on the expression of Ci-pax2/5/8b in Ciona intestinalis. We thank L. Maves and W. Cresko for helpful suggestions and discussion, and A. Amores for providing Hox and Engrailed primers. We thank undergraduate researchers T. Siriphatnaboon and T. Keopuhiwa for help with animal care. We appreciate the work of two anonymous reviewers for helping to improve the manuscript. Complete fosmid sequencing was performed under the auspices of the U.S. Department of Energy, Office of Biological and Environmental Research, in the University of California, Lawrence Berkeley National Laboratory (contract DE-AC03-76SF00098). This material is based on work supported by the National Science Foundation under Grant No. IBN-0345203 and an IGERT grant in Evolution of Development DGE-9972830. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. We thank the Spanish Ministry of Education, Culture and Sports for support for CC (EX2002-0059).

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.06.039.

**References**


Burriil, J.D., Moran, L., Goulding, M.D., Sauersessig, H., 1997. Pax2 is expressed in multiple spinal cord interneurons, including a population of En1 + interneurons that require Pax6 for their development. Development 124, 4493–4503.


